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On the origin of the enthalpy and entropy convergence temperatures in protein folding

(thermodynamics/differential scanning calorimetry/convergence temperatures)

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ABSTRACT **Temperature dependence of the thermodynamics of folding/unfolding for cytochrome *c* has been determined as a function of moderate [0–10% (vol/vol)] concentrations of methanol. Heat capacity change (ΔC_p) for unfolding decreases with increased concentrations of methanol, consistent with a higher solvent hydrophobicity. For a given transition temperature, this effect results in higher experimental enthalpy (ΔH) and entropy (ΔS) changes with increased methanol concentrations. When the enthalpy or entropy data sets obtained at different methanol concentrations are plotted as a function of temperature, they are seen to converge and assume common values around 100°C for ΔH and 112°C for ΔS. These convergence temperatures are similar to those obtained for different proteins in aqueous solution when ΔH and ΔS are normalized with respect to number of residues. It has been previously hypothesized that these convergence temperatures correspond to the temperatures at which the hydrophobic contributions to ΔH and ΔS are zero; the results presented here agree with this viewpoint.**

As early as 1974 Privalov and Khechinashvili (1) noticed that the enthalpy and entropy changes for protein denaturation converged at some characteristic temperatures around 100°C when normalized with respect to the number of residues in the protein. This peculiar behavior has been carefully examined by several authors during the last few years. Baldwin (2) proposed that, at least for the entropy change, this behavior might be related to the hydrophobic effect because of the striking similarity between the convergence temperature for the entropy change in protein folding and the temperature at which the entropy of transfer of liquid hydrocarbons to water is zero. Later, Murphy and Gill (3), using group-additivity thermodynamics and a comparative study of the thermodynamic behavior of proteins and the thermodynamics of dissolution of solid model compounds, proposed that the convergence temperatures for the enthalpy and entropy changes (T^*_H and T^*_S, respectively) correspond to the temperatures at which the apolar contributions to the enthalpy and entropy changes are zero, respectively. Although at the beginning T^*_H and T^*_S were supposed to be identical (1, 4, 5), it later became apparent that T^*_H was centered around 100°C, whereas T^*_S was located near 112°C (6).

Lee (7), by expressing the thermodynamic parameters in terms of the protein buried area, recently proposed that the convergence behavior occurs at that temperature at which the polar and apolar contributions to ΔH and ΔS are equal. However, this view has now been shown (8) to be mathematically equivalent to the view of Murphy *et al.* (3, 4), the difference being in the normalization factor. Murphy *et al.* (3, 4) normalized the protein data with respect to the number of residues in each protein, whereas Lee's analysis (7) is equivalent to a normalization of the data with respect to the total buried area. The two views are equivalent because the family of proteins for which thermodynamic and structural data exist buries a constant polar area but a variable apolar area per residue. Under those conditions, convergence will be observed either at the point at which the apolar contribution is zero (when normalized with respect to number of residues) or at the point at which the polar and apolar contributions are equal (when normalized to the buried area).

In an effort to better define the origin of this convergence phenomenon, we decided to analyze the thermodynamic behavior of a single protein under conditions of different solvent hydrophobicity. If the convergence temperatures are related to the hydrophobic effect, this approach should yield results similar to those obtained with the traditional approach, in which different proteins with variable buried apolar groups have been studied under equivalent solvent conditions. For these studies, the folding/unfolding thermodynamics of cytochrome *c* were measured as a function of pH and moderate methanol concentrations. The effect of moderate concentrations of methanol on protein stability has been attributed to diminished hydrophobic interactions that make more favorable the exposure of buried apolar groups to the solvent (9–12). If this hypothesis is so, this effect must be reflected in a reduced ΔC_p for unfolding and a concomitant reduction of the magnitude of the apolar contribution to the enthalpy change for unfolding. In this paper, as in previous ones (13–15), we follow the convention of Murphy and Gill (3) and define the hydrophobic effect in terms of the thermodynamics associated with the transfer of apolar surfaces from the interior of the protein into the solvent. As such, this definition includes the disruption of apolar–apolar van der Waals' interactions within the protein and the interactions resulting from the exposure of the apolar surfaces to the solvent.

MATERIALS AND METHODS

Cytochrome *c* (horse heart) was purchased from Sigma. Methanol (HPLC reagent grade) was obtained from Baker. The calorimetric studies of the protein folding/unfolding transition were done in 15 mM glycine·HCl buffer (experiments in pH 2–3.6) and in 15 mM sodium acetate (experiments in pH 3.6–5.0). These buffers were chosen because they have been used before in precision calorimetric studies of this protein (1, 16). Protein concentrations were measured spectrophotometrically with an extinction coefficient of 29.5 $mM^{-1}\cdot cm^{-1}$ at 550 nm for the reduced form of cytochrome *c* at pH 7.0 (17). Samples were dialyzed against the appropriate buffer for 24 hr. After dialysis, the desired volume of methanol was added to the sample, the pH was checked, and the sample was placed in the calorimeter cell. In the presence of methanol, the reported pH values correspond to the apparent pH values (12).

All differential scanning calorimetric experiments were done with a Microcal (Amherst, MA) MC2 instrument inter-

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faced to a microcomputer for automatic data collection and instrument control. Analysis of the calorimetric data was done with software developed in this laboratory (18). All experiments were done at a scanning rate of 60°C/hr with sample concentrations of 4–5 mg/ml.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry. The transition temperature, T_m, for the folding/unfolding transition in proteins depends on the solution pH. This pH effect has been used extensively to shift T_m and measure the temperature dependence of the thermodynamic parameters associated with these transitions (1). Fig. 1 shows that with moderate methanol concentrations the effect of pH follows the same pattern. Fig. 1 shows the transition excess heat capacity function of cytochrome *c* as a function of pH for different methanol concentrations. In all cases, the transitions were completely reversible, as demonstrated by repeated scans of the same samples. Experiments were done in pH 2.5–5.0 at methanol concentrations of 0, 5, 7, and 10% (vol/vol). Without methanol and at the higher pHs the transitions conformed closely to the two-state mechanism, as judged by van't Hoff-to-calorimetric enthalpy ratios ($\Delta H_{VH}/\Delta H$) close to unity. With methanol and at lower pH values the transitions become broader and deviate from the two-state mechanism, suggesting that methanol stabilizes some partially folded intermediate states. Previously, a similar methanol effect on the transition cooperativity has been reported for ribonuclease A (12, 19). In our laboratory, we have also observed a similar effect for ubiquitin (unpublished results). In this latter case, NMR data have indicated the appearance of a molten globule intermediate under those conditions (20).

It is apparent from the data in Fig. 1, that for equivalent T_m values the calorimetric enthalpy change (area under curve) becomes larger when the methanol concentration is increased. Additional experiments at a constant pH of 4.0 and methanol concentrations up to 50% (data not shown) reveal that T_m decreases more or less linearly with methanol concentration [T_m = {67.23 − 0.679 (% MeOH)}°C with a regression coefficient of −0.998]. Despite the decrease in T_m, ΔH increases as a function of methanol concentration up to a concentration of ≈20–25%. At higher concentrations ΔH decreases continuously until it cannot any longer be detected at concentrations >60%. This same behavior has been observed before by Velicelebi and Sturtevant (11) for lysozyme in water/alcohol mixtures. In that case also, ΔH increases up to ≈20% methanol and then decreases.

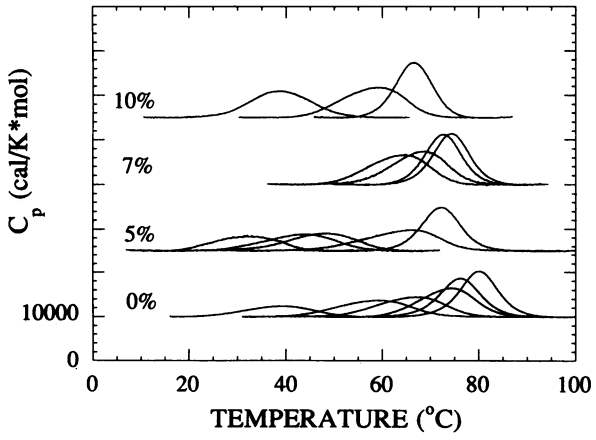

FIG. 1. Transition excess heat capacity versus temperature for cytochrome *c* as function of the apparent pH for different methanol concentrations. These curves were obtained after scan rate and concentration normalization, followed by baseline subtraction, as described in ref. 18. The pH values for each section are (from left to right) as follows: for 0% (vol/vol) methanol, 3.0, 3.2, 3.5, 4.0, and 5.0; for 5% (vol/vol) methanol, 2.8, 3.0, 3.2, and 4.0; for 7% (vol/vol) methanol, 3.3, 3.7, 4.2, and 4.8; for 10% (vol/vol) methanol, 2.8, 3.3, and 4.0.

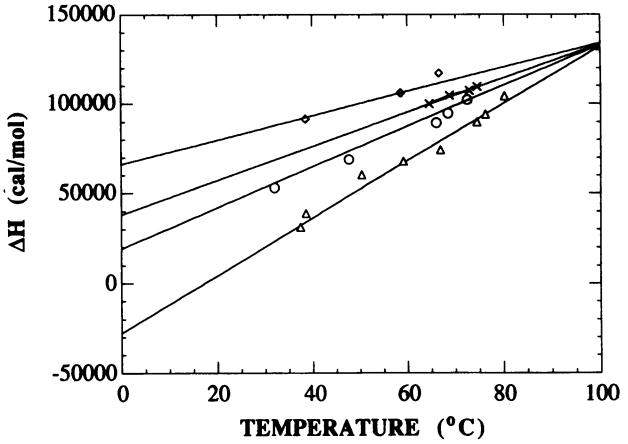

FIG. 2. Temperature dependence of the enthalpy change obtained at different methanol concentrations. Solid lines are the calculated lines obtained by global least-squares analysis in terms of Eq. 1. Methanol concentrations (vol/vol) are indicated as follows: △, 0%; ○, 5%; ×, 7%; and ◇, 10%.

The thermodynamic parameters associated with the excess heat capacity curves in Fig. 1 are shown in Figs. 2 and 3. These data make apparent that, at all methanol concentrations studied, the ΔH for the transition increases as a function of the transition temperature, as predicted for a transition characterized by a positive ΔC_p. The effect of ΔC_p is also evident in the temperature dependence of the entropy change. The ΔC_p for the transition is maximal in the absence of methanol and decreases monotonically with increased concentrations of methanol. In the absence of methanol, ΔC_p is equal to 1.5 kcal/K·mol in excellent agreement with previously determined values (1, 16). ΔC_p decreases linearly with methanol (within the concentration range studied) with a slope of −80 ± 11 cal/K·mol·% MeOH and a regression coefficient of −0.987. Previously, Velicelebi and Sturtevant (11) also found a linear dependence of ΔC_p for lysozyme on the concentration of methanol, ethanol, and 1-propanol.

The most striking feature of the data in Figs. 2 and 3 is the convergence of the enthalpy and entropy changes at temper-

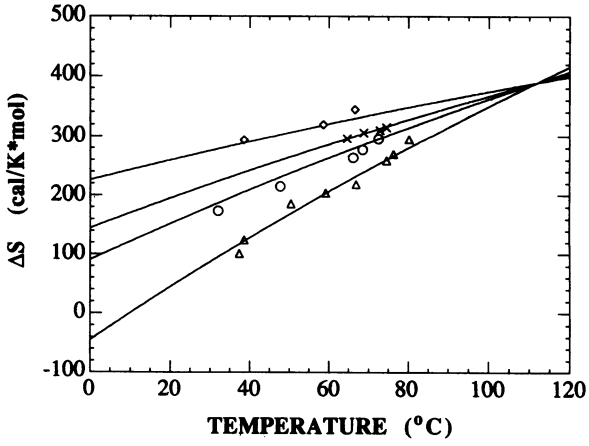

FIG. 3. Temperature dependence of the entropy change at different methanol concentrations. Solid lines are the calculated lines obtained by global least-squares analysis in terms of Eq. 2. Methanol concentrations (vol/vol) are indicated as follows: △, 0%; ○, 5%; ×, 7%; and ◇, 10%.

Table 1. Least-squares analysis of enthalpy and entropy convergence for cytochrome *c*

T^*_H, °C	T^*_S, °C	ΔH^*, cal/mol	ΔS^*, cal/K·mol	ΔC_p, cal/K·mol	$\partial\Delta C_p/\partial$% MeOH, cal/K·mol·% MeOH
101.9 ± 2	111.9 ± 2	135 ± 5	388.8 ± 2	1468 ± 160	−87.0 ± 7

The entire sets of enthalpy and entropy data were fitted separately to Eqs. 1 and 2, as described in text. Values and errors for ΔC_p and $\partial\Delta C_p/\partial$% MeOH are the combined results for the enthalpy and entropy data sets.

atures around 100°C and 112°C, respectively. These temperatures are similar to the convergence temperatures observed when the thermodynamic parameters for different globular proteins (normalized with respect to number of residues or to molecular weight) are plotted as a function of temperature. Also, if ΔH at some reference temperature T_R is plotted versus ΔC_p, the slope is equal to $(T_R - T^*_H)$ (3). Analysis of the cytochrome *c* data by using this procedure yields a T^*_H of ≈103°C. Furthermore, analysis of the lysozyme data obtained at moderate propanol concentrations (<10%) by Velicelebi and Sturtevant (11) yields a T^*_H value of 101°C, also very close to the expected convergence temperature for the enthalpy change.

Thermodynamic Analysis. To test whether the entire set of enthalpy and entropy data could be represented by a single set of thermodynamic parameters, the following equations were used:

$$\Delta H(T) = \Delta H^* + \left(\Delta C^\circ_p + \frac{\partial \Delta C_p}{\partial \%\ \text{MeOH}} \times \%\ \text{MeOH}\right)(T - T^*_H) \quad [1]$$

$$\Delta S(T) = \Delta S^* + \left(\Delta C^\circ_p + \frac{\partial \Delta C_p}{\partial \%\ \text{MeOH}} \times \%\ \text{MeOH}\right)\ln(T/T^*_S), \quad [2]$$

where T^*_H and T^*_S are the convergence temperatures for the enthalpy and entropy changes, respectively, and ΔH^* and ΔS^* are the values of the enthalpy and entropy changes at those temperatures. In the above thermodynamic relations, ΔC_p has been assumed to vary linearly with the methanol concentration, as suggested by the experimental data. Strictly speaking, ΔH^* and ΔS^* are a function of pH; however, their variation with pH is very small and within the error of each experimental measurement. For ΔH^*, the protonation enthalpies are almost completely compensated for by the buffer (1, 16). For ΔS^*, the entire range of experimentally observed T_m values is generated by a change of approximately ± 2% in ΔS^*.

Global analyses of the entire sets of enthalpy and entropy data were performed by nonlinear least-squares technique to obtain the best set of parameters that fits the data. Results of the analysis are summarized in Table 1. The solid lines in Figs. 2 and 3 were calculated with Eqs. 1 and 2 with the parameters in Table 1. The SD for the fit is 0.8 kcal/mol for the enthalpy change and 2.3 cal/K·mol for the entropy change. The error for each of the fitting parameters was calculated by a support plane method (21, 22) for both the enthalpy and entropy fits. The reported errors for ΔC_p and $\partial\Delta C_p/\partial$% MeOH in Table 1 are the combined errors obtained for both data sets.

It is apparent from the above analysis that the convergence behavior observed as a function of methanol resembles the convergence behavior obtained from the analysis of different proteins in aqueous solutions. In both cases, the convergence temperatures for both the enthalpy and entropy changes are the same within error. The best current estimates for the analysis of different globular proteins in aqueous solution are 100 ± 6°C for T^*_H and 112 ± 1°C for T^*_S (for review, see ref. 13), compared to the values of 101.9 ± 2°C and 111.9 ± 2°C obtained from the methanol dependence of the thermodynamic parameters for cytochrome *c*.

Dissection of Energetic Contributions to Stability. While in the past the positive ΔC_p for protein unfolding was solely attributed to the exposure of buried apolar groups to water upon denaturation, polar groups are now known to also contribute to this effect, although with a negative sign (6, 23). The best current estimates based upon solid model compound dissolution and analysis of the existing protein data base are $\Delta C_{p,pol} = -0.26$ cal/K·mol·Å^2 and $\Delta C_{p,ap} = 0.455$ cal/K·mol·Å^2 (6, 8, 13). These values, in conjunction with polar and apolar accessible-area calculations can be used to estimate the polar and apolar contributions to ΔC_p (6, 8, 13–15). Upon denaturation cytochrome *c* exposes to the solvent 3850 Å^2 of buried polar area and 5420 Å^2 of buried apolar area. This translates into an overall ΔC_p value of 1.47 kcal/K·mol in excellent agreement with the experimentally determined values. The calculated apolar and polar contributions are 2.47 and −1.0 kcal/K·mol, respectively.

Fig. 4 represents the temperature dependence of the enthalpy change for the folding/unfolding transition of cytochrome *c*. As mentioned before, the value estimated at the convergence temperature by global least-squares analysis is 135 ± 5 kcal/mol. The average ΔH^* value at the convergence temperature obtained from the analysis of different globular proteins in aqueous solution is 35 ± 1 kcal/mol-Å^2 of buried polar area (8, 13). This value predicts a ΔH^* for cytochrome *c* of 134 kcal/mol in close agreement with the experimental value. In Fig. 4, the calculated polar and apolar contributions to the enthalpy change are shown. The solid lines were calculated by using the values discussed in the previous paragraph. For illustration purposes, the expected effect of 7% (vol/vol) methanol is also shown.

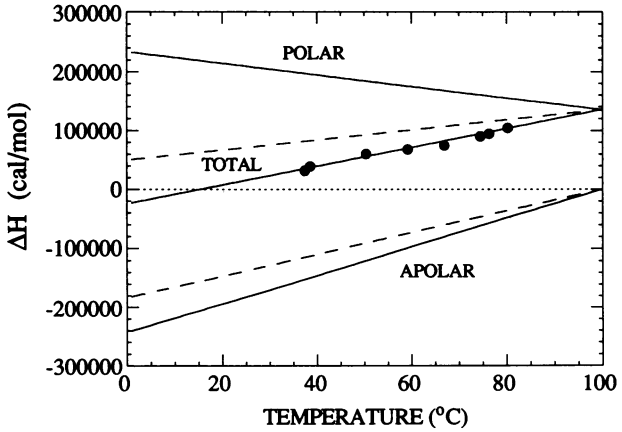

FIG. 4. Calculated polar and apolar contributions to the total enthalpy change for the folding/unfolding of cytochrome *c* (see text for details). The apolar contribution to the enthalpy change is assumed to be zero at the convergence temperature (100°C). At this temperature, the measured enthalpy contains only polar contributions. The expected effect of methanol is illustrated by the dashed lines for the 7% case.

The existence of a convergence temperature with a mixed solvent system strongly suggests that the effect of methanol on the energetics of the transition is due primarily to its effect on ΔC_p. Any effect of methanol on the intrinsic enthalpy of hydrogen bonding or other interactions would have been reflected in either the absence of a convergence temperature or a shift of this temperature away from the value obtained for the set of globular proteins in aqueous solution. In this respect, Susi and Ard (24) have shown in the past that the enthalpy change for the interaction of amide groups through hydrogen bonding is the same in water and in methanol. Also, the fact that a similar convergence temperature is seen for other proteins (11) in different water/alcohol mixtures is consistent with this idea.

It is also important to note that, within the experimental error, ΔC_p decreases linearily with methanol concentration. This effect has been observed by Velicelebi and Sturtevant (11) for lysozyme as a function of the concentration of methanol, ethanol, and 1-propanol. From a purely mathematical point of view (see Fig. 4) several effects are, in principle, consistent with the observed constant ΔH^* and variable ΔC_p: rotation of ΔH_{pol}, ΔH_{ap}, or both around the axis defined by T^*_H; equal vertical displacement of ΔH_{pol} and ΔH_{ap}; or a combination of both. It seems, however, very unlikely that the presence of the organic solvent will affect all parameters and that, somehow, all those effects will cancel to produce a constant ΔH^*. If ΔH_{pol} is not significantly affected by methanol, as suggested by the data on model compounds, then a vertical displacement is ruled out due to the condition ΔH^* = constant. Although, in principle, methanol can affect both $\Delta C_{p,pol}$ and $\Delta C_{p,ap}$, the lack of an effect on ΔH^* argues against a significant effect of methanol on $\Delta C_{p,pol}$. If methanol is assumed to affect only $\Delta C_{p,ap}$, then a linear extrapolation of the calorimetric data predicts that at ≈20% methanol ΔC_p will be zero and that at ≈28% methanol the apolar contribution to ΔC_p must be zero.

CONCLUSIONS

Our data provide strong support to the hypothesis that the convergence temperatures for the enthalpy and entropy changes in protein denaturation are related to the hydrophobic effect. The addition of moderate concentrations of methanol presumably increases the hydrophobicity of the solvent, resulting in a lower ΔC_p for the exposure of apolar groups to the solvent. This effect can be interpreted in terms of a preferential binding (25) or a solvent structure formalism (9, 10, 12). The net observed result would be a decrease in the magnitude of the apolar contribution to the enthalpy change of denaturation within the experimental temperature range. At the convergence temperature, however, the enthalpy change is independent of the relative hydrophobicity of the solvent. If the effect of methanol is due mainly to a decrease in the apolar contribution to ΔC_p, as appears to be indicated by the data, then a convergence temperature is expected at the temperature at which the apolar contribution to the enthalpy is zero or a constant independent of MeOH concentration within the range studied. This situation is illustrated in Fig. 4. For a family of proteins in aqueous solution, on the other hand, convergence of the residue-normalized enthalpy change is seen when the proteins bury a constant polar area per residue and a variable apolar area per residue. Taken together, the two approaches suggest that, at T_H^*, the apolar contribution to the enthalpy is most likely zero since ΔH^* is independent of the apolar area buried per residue.

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